Laboratory Culture, Metamorphosis and Development of *Aplysia brasiliana* Rang, 1828

(Gastropoda : Opisthobranchia)

BY

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(1 Text figure)

INTRODUCTION

Because of their increasingly important use in neurophysiological and behavioral studies (Kandel, 1976) select species of opisthobranch mollusks have recently become the subjects of expanded field and laboratory culture studies (for review, see Thompson, 1976 and Switzer-Dunlap & Hadfield, 1977). Laboratory culture techniques have been established for *Aplysia californica* Cooper, 1863 (Kriegstein, Castellucci & Kandel, 1974) and *A. juliana* Quoy & Gaimard, 1832 and *A. dactylomela* Rang, 1828 (Switzer-Dunlap & Hadfield, *op. cit.*). Kriegstein (1977) presents detailed descriptions of development in *A. californica*. Due to its geographical location, this laboratory has greatly expanded its interest (Blankenship & Coggeshall, 1976) in the study of *A. brasiliana* Rang, 1828. This species is common to the South Texas Coast (Strenth & Blankenship, 1977) and adults are easily maintained in the laboratory. The results of this study provide simple culture techniques along with descriptions of metamorphosis and post-larval development of this scientifically useful marine gastropod.

METHODS

Natural sea water is used in the culture of veligers and post-metamorphic juveniles. This is collected, adjusted to 30 ppt., filtered through a 0.2 μm membrane filter, and stored in carboys until ready for use. Living specimens of *Aplysia brasiliana* were collected from South Texas and maintained in the laboratory in artificial sea water of 28 to 30 ppt. at 18° C to 22° C. They were fed daily on a diet of "Laver" (commercial name of dried *Porphyra*). Eggs laid by laboratory-held specimens of *A. brasiliana* hatch in about 8 to 10 days.

Prior to hatching, eggs are removed from the holding tanks and placed in open petri dishes containing natural sea water. This facilitates examination by microscope and provides a concentrated source of veligers following hatching. Water in the petri dish is changed daily until hatching. Preparation dishes (Pyrex No. 3250, 100×80 mm) are used for the culture of the free-swimming veligers during the remainder of their larval development. Several weeks prior to their use as containers in the culture process, preparation dishes must be readied, or conditioned, for acceptance of veligers. This process involves the adding of 300 mL of paper-filtered natural sea water to each dish, replacing the cover and placing it in the laboratory where it receives normal room light. This procedure facilitates growth of a coating of diatoms or algae, or both, on the inner surface of the dish. Prior to the transfer of veligers, the preparation dish is emptied of old water, flushed a couple of times with filtered sea water, and filled with 300 mL of freshly aerated membrane-filtered sea water. Care is taken to prevent removal of the coating on the inner surface of the preparation dish. Fifty to 150 free-swimming veligers are transferred by means of glass pipettes into the preparation dish which is placed within 5 to 10 cm of a fluorescent lamp. Optimal results were obtained with light entering from the side of the culture containers. Cultures are maintained at temperatures of 21° C to 25° C and glass tops are retained on the preparation dishes at all times except for feeding or removing specimens for examination.

Feeding is accomplished by the addition of the unicellular algae *Isochrysis galbana* to the culture. Unialgal cultures of this species are maintained at peak or near-peak growth of 1 to 2 million cells per milliliter (Provasoli, 1968). Prior to feeding, 200 mL of *Isochrysis* culture

is centrifuged at 3800 rpm for 15 minutes. Following centrifugation, the supernatant culture medium is poured off and the concentrated "pellets" of *Isochrysis* cells are resuspended in 100 mL of aerated filtered sea water. This is allowed to stand in 2 centrifuge tubes for 24 hours prior to feeding. Once prepared, these tubes can be used for feeding for up to 3 days. One mL is pipetted from the surface region of this prepared medium and evenly distributed throughout the veliger culture. This gives an approximate density of 5 000 to 10 000 *Isochrysis* cells per milliliter in the culture medium. During feeding the culture water is vigorously mixed by use of the pipette. Feeding and mixing is done once daily.

Once initiated, the veliger culture requires little maintenance other than daily feeding and mixing. Cultures have been maintained for up to 70 days without water change. Survival rates by use of this method may vary, but can be easily maintained near the 50% level with minimal care. Two major sources of mortality account for this reduced survival rate. The veliger larva is pelagic in its natural environment and lack of stimulation by flowing current in this culture method apparently results in a settling out of moderate numbers of veligers during the first 7 to 10 days following culture initiation. These become trapped in the substrate, cease natural feeding activities, and eventually perish. The air-water interface accounts for the other major source of veliger mortality. Once a fast swimming veliger comes in contact with the interface, it becomes trapped on the water surface. Loss of veligers at the interface appears directly related to the density of veligers in culture; proportionally greater losses occur in crowded cultures. This problem is somewhat reduced by maintaining the water depth at approximately 5 cm. In addition, loss of veligers at the air-water interface can be minimized by routine daily examination beneath a dissecting microscope during which time trapped veligers may be resubmerged by dropping water from a pipette. The use of compounds (Hurst, 1967; Switzer-Dunlap & Hadfield, 1977) designed to reduce the surface film was found unnecessary in the culture of *Aplysia brasiliana* veligers. This moderate veliger loss appears more than acceptable in exchange for removal of complicated sealed systems (Kriegstein *et al.*, 1974) or mechanical stirring devices, or both, designed to eliminate these losses.

Once individual veligers exhibit visual signs indicative of attainment of metamorphic age they are transferred to a covered glass petri dish (Pyrex No. 3160, 60×15 mm) of filtered sea water containing *Isochrysis*. A small tuft of the red alga *Callithamnion* from South Texas is added and veligers are observed for indications of metamorphosis. [Although this alga conforms to the description of *Callithamnion byssoides* Arnott *in* Hooker given by Edwards (1970), Dr. Clinton J. Dawes of the University of South Florida has identified the material (personal communication) as *Callithamnion halliae* Collins.] Metamorphosis was also induced upon exposure of veligers to at least one species of *Polysiphonia*; successful metamorphosis, however, was quite low and results were not encouraging. Veligers under observation are transferred to a petri dish containing fresh sea water and *Callithamnion* every 2 days until feeding is observed. Once feeding begins, the juvenile specimens are transferred daily to fresh water and algae until specimens are large enough to place in small holding tanks. Natural seaweed is fed for 6 to 8 weeks following metamorphosis. At this time specimens may be induced to feed on the commercially available dried seaweed.

RESULTS AND DISCUSSION

Eggs laid by laboratory held *Aplysia brasiliana* exhibit wide variation in color. While egg masses are generally dark green to light brown in color, both red and yellow strings have been observed. The egg strand is approximately 1 mm in diameter and usually coiled upon itself during the laying process. There are approximately 150 capsules per cm and, though variable, each capsule contains about 24 eggs. Capsules at the beginning or end of an egg string are often empty or contain a reduced number of eggs. Rotation of individual eggs is observed within 4 days following laying. At 8 to 10 days well developed veligers are observed actively moving about in the capsules. This is shortly followed by disintegration of the egg case resulting in the release of large numbers of free swimming veligers.

Veligers are approximately 140 μm in diameter at hatching. They are active in their movements and exhibit positive phototaxis. Growth is progressive and by 30 to 40 days specimens attain a diameter of approximately 325 to 375 μm. At this time individuals exhibit a decrease in swimming activity associated with frequent propodal extension or attachment, or both, to the culture container. This is a visual indicator that the veligers have attained the size and age necessary for initiation of metamorphosis. At this time exposure to a freshly collected tuft of the red seaweed *Callithamnion* will trigger the metamorphic process. Veligers will attach and crawl about on the algae by means of the propodium. Individual veligers may remain permanently attached to the seaweed or may briefly remain attached, withdraw and later return. Successful metamorphosis has been attained in 80% of the specimens retained in culture for 55 days.

The onset of metamorphosis is signaled by propodial attachment followed within 24 hours by loss of the velar cilia and retraction of the velar folds (Figure 1a). This is

accompanied by the appearance of 5 to 10 red pigment spots on the upper right surface of the veliger shell (Figure 1b). The number of these pigment spots will eventually increase to about 25 to 30. Loss of the velar cilia terminates the larval ability to filter-feed and swim. This is followed by approximately 4 days during which time the veliger remains attached to the algae. Individuals at this stage exhibit intermittent periods of limited crawling activity. Dislodgement of the veliger during this quiet phase will reveal in some specimens the presence of a small attachment thread. At approximately 2 to 3 days following settlement muscular movement of the radula sac similar to that exhibited during feeding is observed. This is followed at about 5 days by actual feeding.

The onset of feeding is followed within 48 hours by loss of the operculum. This is accompanied by a rapid increase in size as well as an increase in red pigmentation of the exposed body surface. Along each side of the head a depigmented tract extends anteriorly from the margin of the adult shell to include the outer distal area of the cephalic tentacle (Figure 1c)). In these tracts are located the eyes as well as randomly scattered large white pigment granules. These white granules are also common along the upper margin of the newly forming parapodia. The mid-

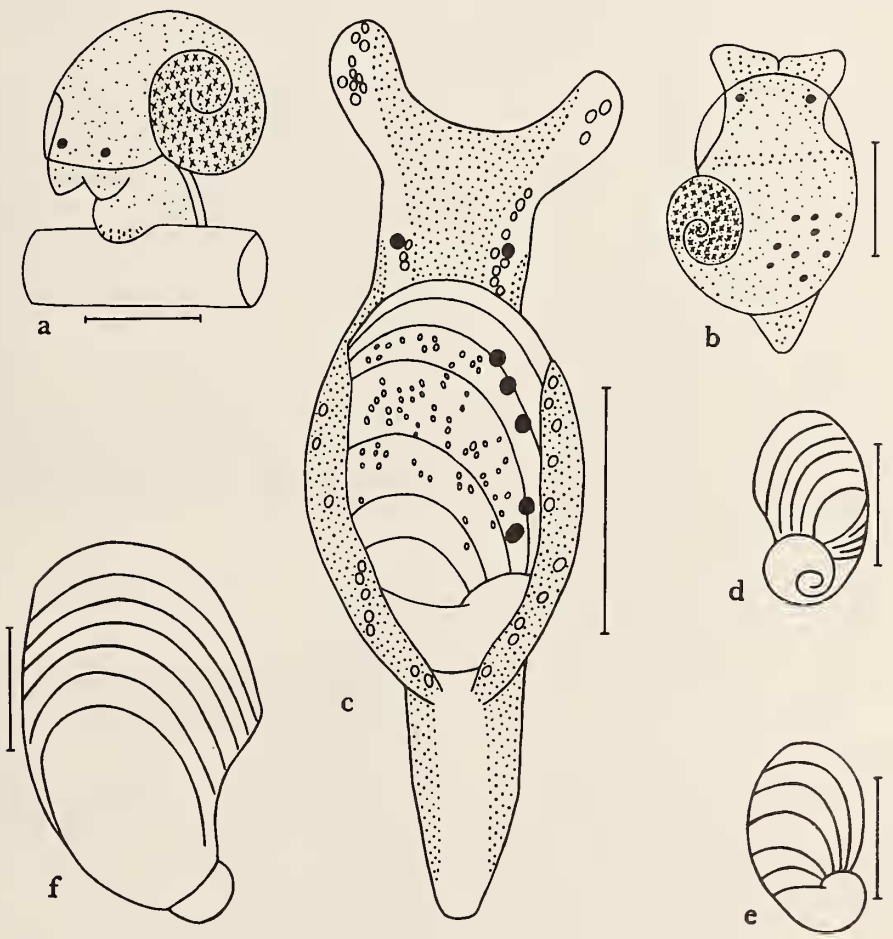

Figure 1

1a. Larval stage of *Aplysia brasiliana* 24 hours following the initiation of metamorphosis

1b. Post-metamorphic stage at 36 hours following the onset of feeding

1c. Juvenile specimen at 5 days post-feeding

1d. Ventral view of early developing adult shell with protoconch attached

1e. Dorsal view of Figure 1d

1f. Dorsal view of developing shell of 10 day post-feeding juvenile

Scale of a and b is 0.2 mm. Scale of c, d, e, and f is 0.5 mm

dorsal surface of the tail (Figure 1c) also lacks red pigmentation. Three to 10 large dark pigment spots begin to form just beneath the surface of the right margin of the newly forming adult shell (Figure 1c). These cells represent the initial formation of the ink gland of the adult.

Although growth rates of individual specimens exhibit a wide degree of variability, most specimens attain 1 mm in total length by 4 days following the onset of feeding. Two small budlike protuberances just posterior to the eyes make their appearance at about 7 to 8 days post-feeding. These structures will enlarge and form the rhinophores of the adult. By about 10 days the dorsal margins of the rapidly enlarging parapodia come in contact and are able to completely enclose the mantle cavity. Specimens are 2.5 to 3 mm in length at this stage and will undulate the parapodia in an effort to assist locomotion along the algae upon which they are feeding.

By 14 days following initiation of feeding, specimens attain lengths of up to 8 mm. The parapodia are greatly enlarged and are folded upon one another to form 2 dorsal siphons. Swimming may be induced by removal of specimens from the algae upon which they are feeding. The distal tips of the now stalk-like rhinophores are cup-shaped. The seminal groove is first noted at this stage. It is poorly developed and only faintly visible. By the 18th day specimens attain a total length of 1.5 cm. At this time the mantle foramen occupies only about one half of the surface area of the adult shell. By 40 days specimens are 3 cm in length and can be induced to feed on the commercial dried seaweed "Laver." Sexual maturity is usually attained by 2 to 3 months post-metamorphosis depending upon food availability and individual growth rate. The above sequence of morphological changes in post-metamorphic development is given for those specimens exhibiting optimal growth rates. Many specimens will be observed to develop less rapidly. Food availability appears to be a major factor in determining individual growth rates.

The development sequence observed in *Aplysia brasiliana* during this study is very similar to that noted for *A. californica* (Kriegstein *et al.*, 1974) and *A. juliana* and *A. dactylomela* (Switzer-Dunlap & Hadfield, 1977). Major differences are noted in selection of different algal species required as triggering agents for the initiation of metamorphosis.

SUMMARY

Eggs laid by laboratory-held specimens of *Aplysia brasiliana* hatch in about 8 to 10 days. Newly hatched veligers were cultured in filtered sea water at temperatures of 21° C to 25° C. Cultures were maintained at salinities of 28 to 30 ppt. and fed the unicellular algae *Isochrysis galbana*. Metamorphosis was induced as early as 30 days post-hatching upon exposure to freshly collected specimens of the red seaweed *Callithamnion*. Specimens could be retained in culture for as long as 70 days prior to metamorphosis. Propodial attachment, loss of the velar cilia, and retraction of the velar folds signal the onset of metamorphosis. Feeding begins at 5 days following settlement. This is followed by a rapid increase in growth rate. Sexual maturity is attained at about 2 to 3 months post-metamorphosis, depending upon food availability.

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